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TITLE: Redox Abnormalities as a Vulnerability Phenotype for Autism and Related Alterations in CNS Development

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14. ABSTRACT
We hypothesize that low systemic redox potential (GSH/GSSG; cysteine/cystine) reflects a vulnerability phenotype that is associated with regressive autism and is predictive of the risk of developing autism. The redox vulnerability phenotype is associated with epigenetic alterations in primary immune cells that may be reversible with restoration of intracellular redox potential. The hypothesis predicts that children with regressive autism and high risk (developmentally-delayed) children who are subsequently diagnosed with autism will exhibit lower redox potential compared to age-matched unaffected control children. It also predicts that low redox potential from these children will be associated with epigenetic modifications in DNA methylation and histone acetylation/methylation that are reversible with treatment to restore redox potential. In Aim 1 we will determine whether redox potential in immune cells can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism. We will also evaluate immune redox potential from high risk developmentally delayed children to determine whether redox status is predictive of subsequent development of autism. In Aim 2, we will determine whether immune cells from autistic children are associated with altered cytokine patterns, macrophage/T cell DNA methylation, and chromatin histone methylation compared to control children

15. SUBJECT TERMS
None provided.

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PROGRESS REPORT 2011
2010-2011 Accomplishments

Project 1 PI: S. Jill James PhD

INTRODUCTION:

Based on our preliminary studies, we hypothesized that children with autism spectrum disorders (ASD) have a more oxidized metabolic status than normal children. The goal of Aim 1 of this project is to better define the functional implications of redox abnormalities associated with autism and to study the predictive potential of the GSH/GSSG redox ratio as a biomarker for autism. The goal of Aim 2 is to determine whether targeted treatment to increase redox potential will alter cytokine balance and epigenetic alterations in primary immune cells from children with ASD. A summary of our progress to date is summarized below in the body of the report *following the format of Project 1 SOW* and is accompanied by data generated this year with interpretation of these results.

BODY:

***Aim 1*: Determine whether redox potential in plasma can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism.**

a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing NIH grant (1RO1HD051873) (months 1-4) **Done**

b) Biomarkers for regressive autism: Selection of children with 50 regressive autism, 50 infantile autism and 50 age-matched control boys; Selection of 30 children with developmental delay (DD) with diagnosis of autism; 30 children with DD without autism; 30 age-matched control children (on-going years 1-3). To date we have recruited 24 children with sudden onset regression, 35 children with infantile autism and

Progress: Below we show that children identified with regressive autism have elevated levels of 3-nitrotyrosine (Figure 1), a biomarker of protein oxidative damage and lower levels of total glutathione (Figure 2) compared to age-matched control children. Additionally, we show in Figure 3 that elevated nitrotyrosine is positively associated with levels of oxidized glutathione (GSSG) and negatively associated with GSH levels (Figure 4) in children with sudden onset regression.

Figure 1

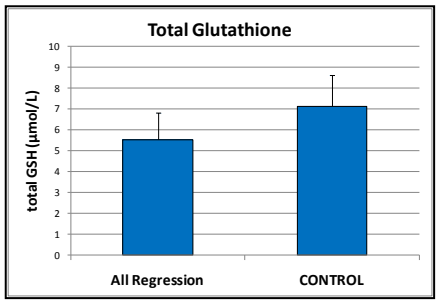


Figure 2

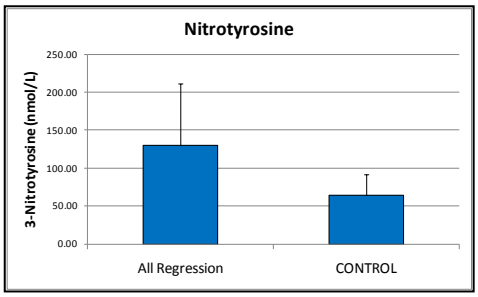


Figure 3

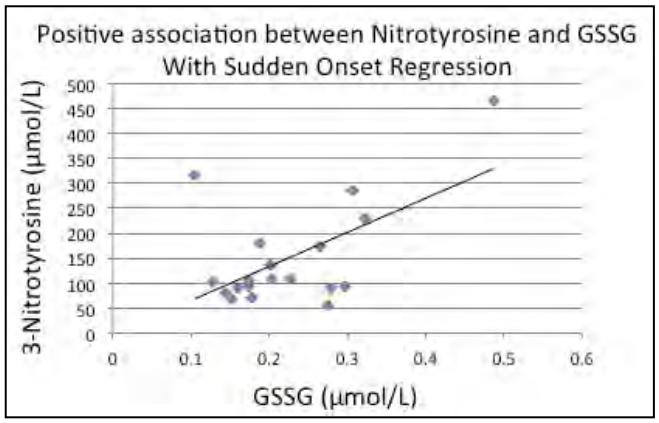
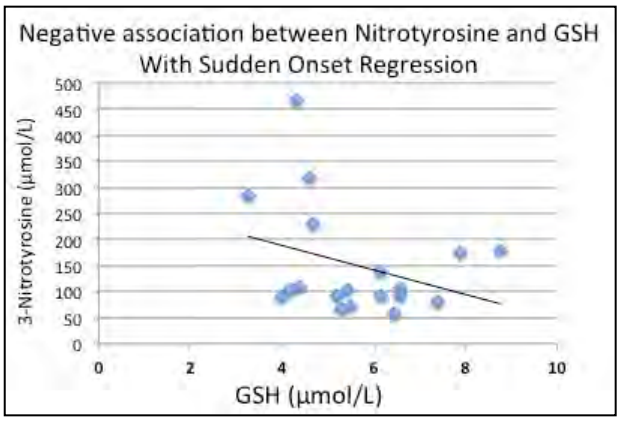
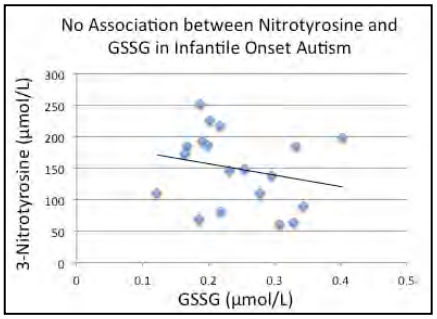


Figure 4



In contrast, children with Infantile Onset autism did not show significant association with oxidized GSSG.

Figure 5



Interpretation: Compared to unaffected control children, the autistic children who underwent regression had lower levels of plasma glutathione which would be consistent with a more oxidized internal environment. Lower mean levels of glutathione were accompanied by an increase in nitrotyrosine, a biomarker of oxidative protein damage. The positive correlation between nitrotyrosine and oxidized GSSG and the negative correlation between GSH and nitrotyrosine in children who underwent sudden regression compared to those who presented with infantile autism suggests that a predisposition to chronic oxidative stress may be an underlying mechanism contributing to sudden regression. Together these data suggest that the decrease in antioxidant "capacity" is accompanied by an increase in oxidative damage in children with autism. The functional consequence, if any, cannot be determined from this data but it indicates that the more oxidizing conditions are associated with altered macromolecular structure.

In Figures 6-8, we divided the regression cohort into "sudden" regression and "gradual" regression and find that both subgroups have decreased plasma levels of glutathione and increased nitrotyrosine protein oxidative damage relative to unaffected controls (Figures 6 and 7). Interestingly, low levels of SAM/SAH, an indication of reduced methylation capacity was associated with a significant decrease in global DNA methylation (Figure 8).

Figure 6

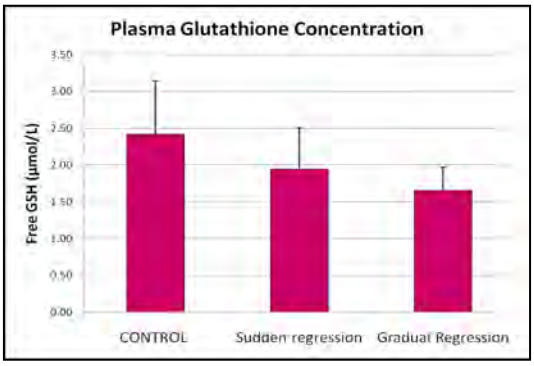


Figure 7

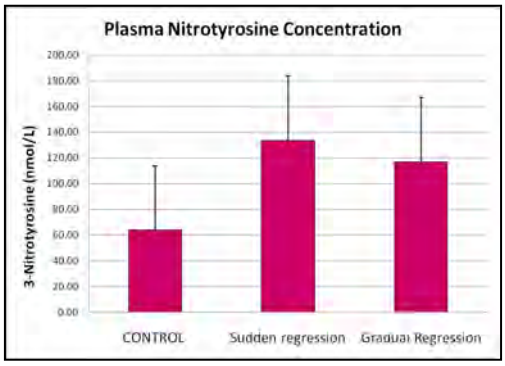
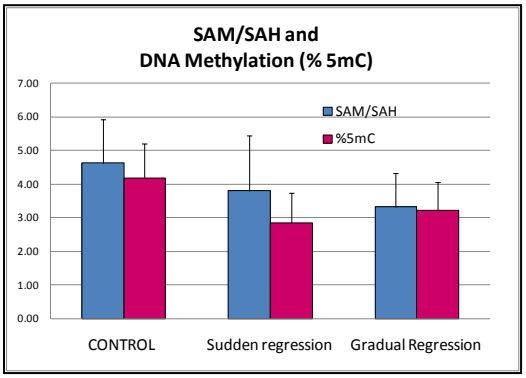


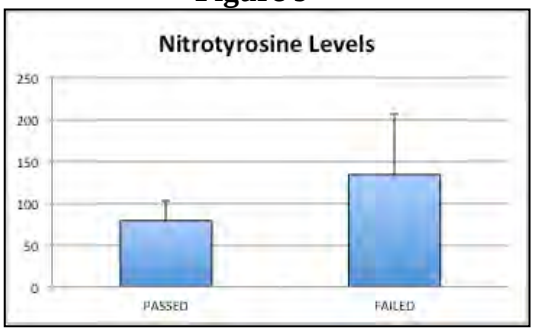
Figure 8



c) Predictive biomarkers in toddlers (age 18-30 months) with developmental delay who fail a standardized autism screening test (MCHAT) and are at high risk of developing autism. We have used extracts from plasma and primary cells to identify redox-related predictive biomarkers of autism in newly recruited cases and controls (ongoing; years 1-3)

Progress: Because extracts from frozen RBC pellets proved to be unstable and not reproducible, we have been collecting data on fresh primary leukocytes from newly recruited patients. Preliminary results are presented below comparing plasma nitrotyrosine levels in 30 developmentally delayed children who failed the MCHAT (high risk) with those that passed the M-CHAT (low risk) .The data in In Figure 9 below, nitrotyrosine levels are shown to be significantly increased among the high risk children who failed the MCHAT compared to those who passed. None of the other biomarkers evaluated were significantly different between children who passed or failed the MCHAT.

Figure 9



Interpretation: These results are the first evidence of a biochemical marker that could be predictive of subsequent development of autism in children who have been diagnosed with developmental delay.

e) HLPC-ESI-MS analysis of redox couples GSH/GSSG and cysteine/cystine in blood samples and mouse tissue from Dr. Noble (ongoing; years 1-3).

Progress: The preliminary results for the GSH/GSSG and Cysteine/Cystine redox ratio in 55 blood samples from autistic children and 51 age-matched control children are presented in the table below

Metabolite	Case Children		Control Children		Corrected Z Test	
	n	Mean ± SD	n	Mean ± SD	Difference (95% CI)	P-value
Plasma						
GSH (μM)	38	1.58 ± 0.23	41	1.99 ± 0.22	-0.41 (-0.50, -0.31)	<0.001
GSSG (μM)	38	0.20 ± 0.06	41	0.13 ± 0.04	0.07 (0.05, 0.09)	<0.001
GSH/GSSG	38	8.24 ± 2.20	41	17.14 ± 5.54	-8.73 (-10.52, -6.94)	<0.001
Oxidized GSH (%)	38	0.20 ± 0.05	41	0.11 ± 0.03	0.09 (0.07, 0.10)	<0.001
Cysteine (μM)	41	21.7 ± 4.88	41	21.43 ± 4.08	0.13 (-1.88, 2.14)	0.90
Cystine (μM)	41	29.2 ± 10.6	41	19.26 ± 4.8	9.73 (6.25, 13.2)	<0.001
Cysteine/Cystine	41	0.79 ± 0.18	41	1.14 ± 0.18	-0.33 (-0.41, -0.26)	<0.001
E_h for Cysteine		-106 mV		-111 mV		

Interpretation: Both GSH/GSSG (reflection of *intracellular* redox status) and Cysteine/Cystine (reflection of plasma *extracellular* redox status) were significantly lower in autistic compared to control children. Together

these results suggest that both intracellular and extracellular environments are more oxidized in children with autism.

***Deliverables**:* Two publications:
1. Melnyk S, Fuchs GJ, Schulz E, Lopez M, Kahler SG. Fussell JJ, Pavliv O, Rose S. Seidel L. Gaylor DW, **James SJ**. Metabolic Imbalance associated with methylation dysregulation and oxidative damage in children with autism. ***Journal of Autism and Developmental Disabilities*** (epub ahead of print) **2011**
2. Rose, S, Melnyk, S, Trusty, TA, Pavliv, O, Seidel, S, Li, J, Nick, T and **James SJ** "Intracellular and Extracellular Redox Status and Free Radical Generation in Primary Immune Cells from Children with Autism," ***Autism Research and Treatment***, 2012, (in Press).

The discovery of a more oxidized phenotype among children with regressive autism and/or as a biomarker for the risk for developing autism would provide new insights into the etiology of autism as well as earlier detection and new treatment strategies.

Aim 2: Determine whether targeted treatment to increase glutathione redox potential in autistic children will restore cytokine balance and reverse epigenetic alterations in primary immune cells.

a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing Arkansas Children's Hospital Foundation intervention study (months 1-4) **Done**

b) Sample collection before and after nutritional intervention to increase GSH/GSSG redox in 20 autistic (total 40 samples) and 20 controls in our IRB-approved clinical trial (Ongoing; yrs 1-3)

Progress: We have screened 32 and recruited 15 autistic children into our double-blind placebo controlled study to date. We are unable to break the code until we have 20 children who have completed the trial so we are unable to analyze effect of intervention until we have a total of 20 participants who have completed the study.

c) Purification of monocyte/macrophages and T cells from fresh blood samples and determine intracellular GSH/GSSG status (years 1-3)

Progress: Methodology for macrophage and T cell isolation has been successfully accomplished using monoclonal antibodies and flow cytometry. We are able to obtain 75% pure monocytes and 90% pure T cells. We have measured intracellular glutathione redox ratio in total PBMCs and in purified primary monocytes and T cells from control children and children with autism. The results shown in Figures 10 and 11 indicate that the intracellular GSH/GSSG ratio is lower and the percent oxidized glutathione (GSSG) is increased in PBMCs, purified monocytes and purified T cells from children with autism compared to unaffected control children.

Interpretation: See data in Rose et al paper in Appendix.

1. PBMC from children with autism have a significantly increased concentration of GSSG as well as more oxidized glutathione equivalents and significantly decreased GSH/GSSG compared to unaffected control children
2. Following PMA/ionomycin stimulation, $CD4^+$ T cells from children with autism have significantly decreased GSH and GSH/GSSG, as well as significantly more oxidized glutathione equivalents compared to unaffected control chil
3. Following LPS stimulation, monocytes from children with autism have a significantly increased concentration of GSSG, significantly decreased GSH/GSSG, and significantly more oxidized glutathione equivalents compar to unaffected control children.

Figure 10
Intracellular and Extracellular Glutathione Redox Imbalance in Autism

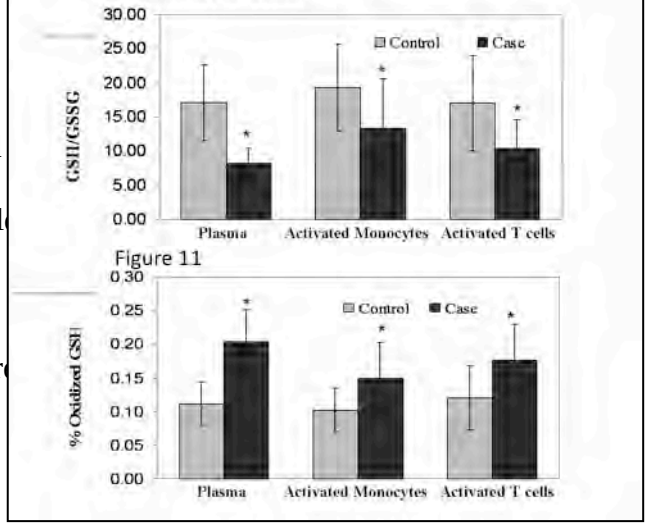


Figure 11

d) Leukocyte global DNA methylation determination using HLPC-ESI-MS technology before and after targeted intervention to increase GSH. (ongoing; years 1-3)

Progress: We have completed DNA extraction and DNA methylation on 96 cases and 81 controls – recruitment is on-going. Results from case and age-matched control children are presented below.

Figure 12: SAM/SAH methylation capacity and global DNA methylation levels in case and control children

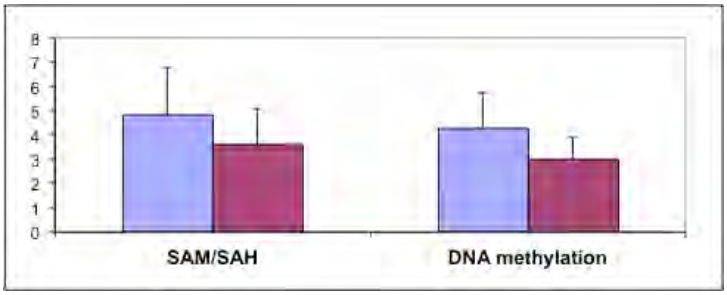
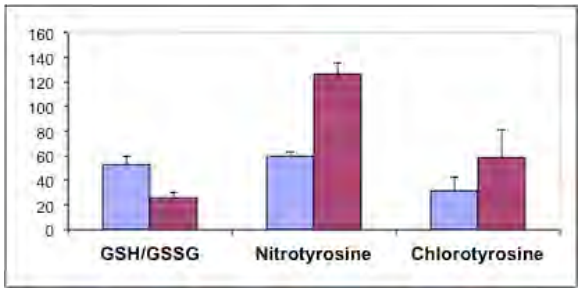


Figure 13: GSH/GSSG redox ratio, and protein oxidative damage (nitrotyrosine and DNA oxidative damage (8-oxo-dG

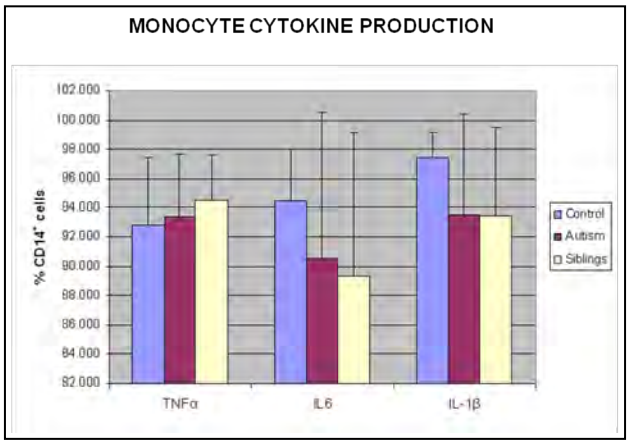


Interpretation: The results presented in Figure 11 suggest that global DNA methylation is significantly decreased in autistic compared to control children and is associated with lower methylation potential (SAM/SAH). Similarly, a decreased in glutathione redox ratio was accompanied by an increase in protein oxidative damage (nitrotyrosine) and DNA oxidative damage (8-oxo-dG). These results suggest that a decrease in methylation and redox potential may have a functional consequence in terms of oxidative damage.

e) Determine intracellular cytokine patterns with flow cytometry in stimulated leukocytes from 30 controls and 30 autistic children before and after intervention to increase GSH (yrs 1-3)

Progress: We have measured intracellular TNFα, IL-1 and IL-6 cytokine production in LPS stimulated monocytes from autistic children, their siblings and unaffected control children.

Figure 13: Intracellular TNF-α cytokine production in LPS-stimulated monocytes



Interpretation: Decreases in IL-6 and IL-1 were observed suggesting a dysregulated immune response to immune stimulation.

f) Determine gene-specific histone acetylation/methylation patterns in T cells using chromatin immunoprecipitation (ChIP) in lymphoblastoid cells from autism and control cell lines (ongoing years 1-3)

Progress: We have worked up methodology of native ChIP and quantification of histone H3K9 and H3K27, and H3K4 methylation and acetylation in the promoter region of the TNF-α gene in lymphoblastoid cells derived from autistic and unaffected control individuals

Figure 14: Ratio of Histone H3K4 (activation) Histone H3K27 (suppression)

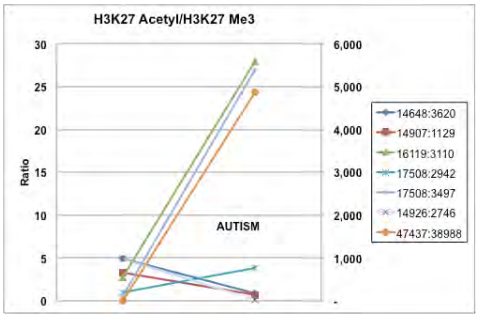
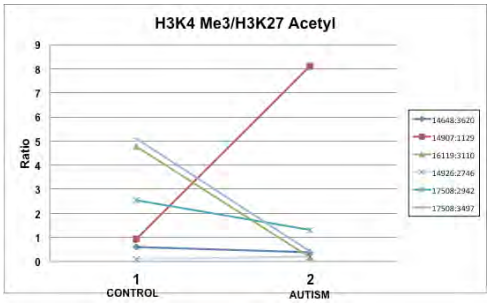


Figure 15: Ratio of H3K27 acetylation (activation) H3K27 methylation (suppression)



Interpretation: Representative results in Figure 14 and 15 are consistent with epigenetic alterations that increase expression of the TNF-a gene in autism cells compared to control cells. We anticipate additional comparisons form at least 6 pairs of case and control cell lines.

Deliverables: We anticipate 2-3 publications in major peer-reviewed journals. We expect that targeted nutritional intervention previously shown to increase plasma GSH/GSSG will increase leukocyte GSH/GSSG and will also restore cytokine balance/epigenetic dysfunction to improve immune function in autistic children. We anticipate that this project will be the first to provide definitive evidence for epigenetic dysregulation of immune function in autism.

Problems encountered and solutions:

1. We were unable to get reproducible results using our stored frozen cell pellets as originally described in the protocol. Instead, we will only be able to measure intracellular GSH/GSSG on fresh primary cells from newly recruited children. Recruitment is ongoing and we anticipate we will have sufficient number of subjects for statistically meaningful results. Because we cannot use previously frozen cells and must rely on newly recruited children, our total samples numbers will be lower than originally projected.
2. We have found that the volume of blood obtainable varies between children. Some children are more difficult to stick and we may only get 3 ml instead of 20ml needed to do full analysis. We have established a priority of assays depending of final volume obtained: PBMCs > monocyte > T cells.
3. We have found that histone purification from primary cells is also dependent on cell volume obtained and has not been reproducible. We have successfully purified histones from lymphoblastoid cell lines obtained from children with autism and unaffected controls. We will do assays on cell lines rather than primary cells to assure reproducibility and meaningful results.

KEY RESEARCH ACCOMPLISHMENTS

- Autistic children who underwent regression had lower levels of plasma glutathione and increased levels of nitrotyrosine, a biomarker of protein oxidation. These results are consistent with a more oxidized internal environment in children with autism.
- The DNA of children who experienced regression was globally hypomethylated compared to unaffected control children. These results are consistent with epigenetic dysregulation.
- Developmentally delayed children who failed a standardized screening test for autism (high risk for autism) had lower plasma glutathione levels and increased nitrotyrosine levels compared to low risk children who passed the screening test.

- The two major redox couples in plasma, GSH/GSSG (reflection of intracellular redox) and cysteine/cystine (extracellular redox) were decreased in children with autism compared to controls.
- Intracellular GSH/GSSG redox was measured in isolated primary PBMCs and purified monocytes and T cells and found to be significantly decreased relative to control children. Intracellular GSSG equivalents were significantly increased in these primary immune cells.
- Biomarkers of protein and DNA oxidative damage (nitrotyrosine and 8-oxo-deoxygaunosine) were increased in children with autism suggesting that the decrease in methylation and redox "capacity" may have a functional consequence in terms of oxidative "damage".
- The production of cytokines IL-6 and IL-1 were decreased in children with autism.
- Methylation of histones in the promoter region of the TNFa cytokine gene were hypormethylated which is consistent with increased expression of this inflammatory cytokine.

REPORTABLE OUTCOMES

1. Publications:

1. Melnyk S, Fuchs GJ, Schulz E, Lopez M, Kahler SG. Fussell JJ, Pavliv O, Rose S. Seidel L. Gaylor DW, **James SJ**. Metabolic Imbalance associated with methylation dysregulation and oxidative damage in children with autism. ***Journal of Autism and Developmental Disabilities*** (epub ahead of print) **2011**
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2. Poster Presentations:

IMFAR 2011:

"Oxidative damage and low glutathione redox status of CNS and peripheral cells in ASD
S. Jill James, Stepan Melnyk, William Starret, Maya Lopez, Jill Fussell, Eldon Schulz, David Gaylor,

SOCIETY FOR NEUROSCIENCE:

"Glutathione Redox Imbalance and Altered TNFα Production in Peripheral Blood Mononuclear Cells from Children with Autism" Shannon Rose, Stepan Melnyk, Oleksandra Pavliv, Timothy A. Trusty, and S. Jill James

NEUROTOXICOLOGY CONFERENCE 2011 (Research Triangle Park. NC): Session Chair and platform presentation: "Oxidative stress in the autism brain"

ELSEVIER CONFERENCE ON AUTISM SPECTRUM DISORDERS: FROM MECHANISMS TO TREATMENT (Arlington VA)

3. Autism Research Institute 2011 Conference: "Maternal Risk factors for autism"

4. Plasma samples have been sent to Co-I Dr. Hepel at SUNY New York

5. Autism Tissue Program: We have **received 13 autism and control matched pairs of frozen brain** tissues from the Autism Tissue Program.

6. NIH R01 has been submitted entitled "Oxidative stress and epigenetic dysregulation in the autism brain" Our data showing histone methylation in the TNF-a promoter was used as preliminary evidence to study similar modifications in frozen brain tissue from autistic and control individuals.

CONCLUSION: Children with autism have a more oxidized intracellular and extracellular microenvironment and a deficit in glutathione-mediated antioxidant/detoxification capacity that makes them more vulnerable to oxidative stress and less able to resolve inflammation.

REFERENCES: None

APPENDICES: One

1. Melnyk S, Fuchs GJ, Schulz E, Lopez M, Kahler SG. Fussell JJ, Pavliv O, Rose S. Seidel L. Gaylor DW, **James SJ**. Metabolic Imbalance associated with methylation dysregulation and oxidative damage in children with autism. ***Journal of Autism and Developmental Disabilities*** (epub ahead of print) **2011**
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J Autism Dev Disord
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ORIGINAL PAPER

Metabolic Imbalance Associated with Methylation Dysregulation and Oxidative Damage in Children with Autism

Stepan Melnyk · George J. Fuchs · Eldon Schulz · Maya Lopez · Stephen G. Kahler · Jill J. Fussell · Jayne Bellando · Oleksandra Pavliv · Shannon Rose · Lisa Seidel · David W. Gaylor · S. Jill James

Abstract Oxidative stress and abnormal DNA methylation have been implicated in the pathophysiology of autism. We investigated the dynamics of an integrated metabolic pathway essential for cellular antioxidant and methylation capacity in 68 children with autism, 54 age-matched control children and 40 unaffected siblings. The metabolic profile of unaffected siblings differed significantly from case siblings but not from controls. Oxidative protein/DNA damage and DNA hypomethylation (epigenetic alteration) were found in autistic children but not paired siblings or controls. These data indicate that the deficit in antioxidant and methylation capacity is specific for autism and may promote cellular damage and altered epigenetic gene expression. Further, these results suggest a plausible mechanism by which pro-oxidant environmental stressors may modulate genetic predisposition to autism.

Keywords Autism · Oxidative stress · Metabolic · Epigenetics · Glutathione · DNA methylation

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The metabolic pathology of autism is relatively unexplored even though metabolic imbalance is implicated in the pathogenesis of multiple other neurobehavioral disorders (Frankenburg 2007; Gysin et al. 2007; Small et al. 2000; Smythies et al. 1997). An abnormal accumulation or deficit of specific metabolites in a defined pathway can provide clues into relevant candidate genes and/or environmental exposures (Zecavati and Spence 2009). Further, the identification of precursor-product metabolite imbalance can inform targeted intervention strategies to restore metabolic balance and potentially improve symptoms of autism. We have chosen to examine key metabolites in the highly-regulated highly polymorphic pathways of folate-dependent one-carbon metabolism in autistic children and the impact of metabolic imbalance on genome-wide DNA hypomethylation and protein/DNA oxidative damage in these children. Because these pathways regulate the distribution of precursors for DNA synthesis (proliferation), DNA methylation (epigenetic regulation of gene expression) and glutathione synthesis (redox/antioxidant defense capacity), the homeostatic balance between these pathways is essential to support the rapid shifts between proliferation, differentiation and cell death that are critical determinants of normal cell programming during pre- and post-natal neurodevelopment.

Previously, we reported that many children with autism have abnormal plasma levels of metabolites in pathways of folate-dependent methionine (transmethylation) and glutathione (transsulfuration) metabolism relative to unaffected age-matched control children (James et al. 2006, 2009a). Specifically, cellular methylation capacity expressed as the mean ratio of the methyl donor S-adenosylmethionine (SAM) to the methylation inhibitor, S-adenosylhomocysteine (SAM/SAH ratio), was significantly reduced in many children with autism. A decrease in the SAM/SAH ratio has been associated with hypomethylation of DNA, RNA,

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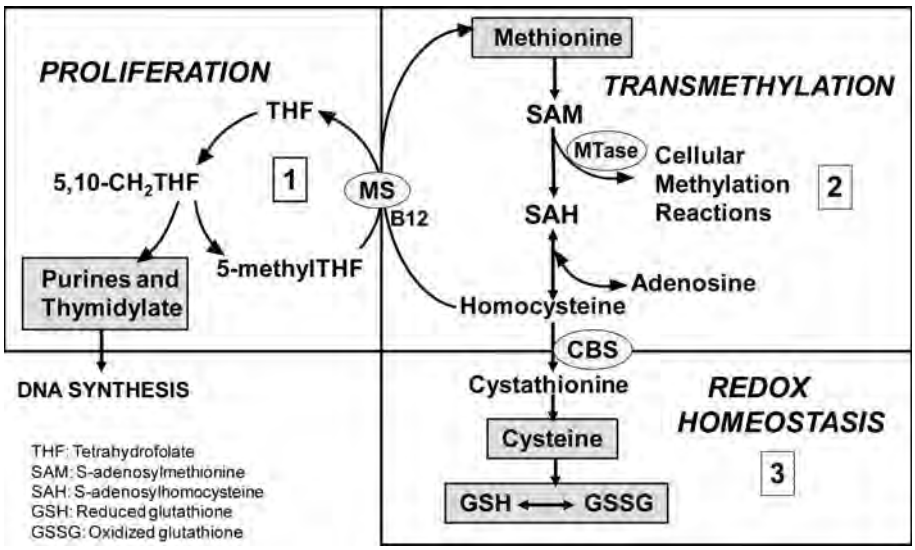

Fig. 1 A diagram of tetrahydrofolate (THF)-dependent methionine transmethylation and transsulfuration for glutathione synthesis. The methionine cycle (transmethylation) involves the regeneration of methionine from homocysteine via the B12-dependent transfer of a methyl group from 5-methyl-tetrahydrofolate (5-CH_3THF) via the methionine synthase (MS) reaction. Methionine is then activated to S-adenosylmethionine (SAM), the methyl donor for multiple cellular methyltransferase (MTase) reactions for the methylation of essential molecules such as DNA, RNA, proteins, phospholipids, creatine, and neurotransmitters. The transfer of the methyl group from SAM results in the demethylated product S-adenosylhomocysteine (SAH). The reversible hydrolysis of SAH to homocysteine and adenosine by the SAH hydrolase (SAHH) reaction completes the methionine cycle. Homocysteine can then be either remethylated to methionine or irreversibly removed from the methionine cycle by cystathionine beta synthase (CBS). This is a one-way reaction that permanently removes homocysteine from the methionine cycle and initiates the transsulfuration pathway for the synthesis of cysteine and glutathione. Glutathione is shown in its active reduced form (GSH) and inactive oxidized disulfide form (GSSG). Vital cell functions dependent on these inter-dependent pathways include proliferation (e.g., immune function, DNA synthesis and repair), methylation (e.g., DNA, RNA, proteins, phospholipids, neurotranmitters and creatine) and redox homeostasis (e.g., cell signaling, detoxification, stress response, cell cycle progression and apoptosis)

proteins, phospholipids and neurotransmitters with functional consequences in terms of gene expression, protein expression, membrane phospholipid composition and dopamine synthesis, respectively (Finkelstein 2007; Miller 2008). The mean level of reduced glutathione (GSH), the major intracellular antioxidant and mechanism for detoxification, was significantly decreased in the autistic children and the oxidized form of glutathione (GSSG) was significantly elevated resulting in a twofold reduction in the GSH/GSSG redox ratio relative to age-matched unaffected control children. Several metabolic precursors for glutathione synthesis were also lower suggesting that GSH synthesis may be insufficient. Decreased GSH/GSSG antioxidant capacity is well known to promote oxidative stress and increase vulnerability to pro-oxidant environmental exposures and oxidative damage. While these data suggest a reduced "capacity" to maintain cellular methylation and antioxidant status, it is important to note that they do not provide evidence for a functional "consequence" of the reduced capacity. In the present investigation, we report the presence of DNA hypomethylation and protein/DNA oxidative damage as potential functional consequences of reduced methylation and antioxidant capacity in these children.

To review, Fig. 1 provides an overview of the three interdependent pathways involved in folate-dependent methionine transmethylation and transsulfuration metabolism with specific biochemical details provided in the legend. Pathway 1 is the folate cycle, Pathway 2 is the methionine transmethylation cycle, and Pathway 3 is the transsulfuration pathway leading to glutathione (GSH) synthesis. The vital importance of these three interconnected pathways is underscored by their essentiality for error-free DNA synthesis (Pathway 1); for cellular methylation capacity (Pathway 2); and for the maintenance of cellular redox homeostasis (Pathway 3). The ratio of the methyl donor S-adenosylmethionine (SAM) to the product inhibitor S-adenosylhomocysteine (SAH) is a reflection of transmethylation efficiency and cellular methylation potential. Tissue-specific gene expression depends on correct promoter region DNA methylation patterns established during embryogenesis and global DNA hypomethylation can result in abnormal gene expression and genomic instability (Reik and Dean 2001; Rizwana and Hahn 1999). The GSH/GSSG ratio reflects the redox potential of the intracellular environment which is critical for maintenance of normal membrane signaling, antioxidant and detoxification capacity (Filomeni et al. 2002; Pastore et al. 2003). In

the present investigation, we evaluated global DNA methylation (as % 5-methylcytosine) to determine whether reduced methylation capacity (↓SAM/SAH) is accompanied by DNA hypomethylation and whether reduced antioxidant/detoxification capacity (↓GSH/GSSG) is associated with an increase in oxidized protein (3-nitrotyrosine) and oxidized DNA adducts (8-oxo-deoxyguanosine) in case children, paired siblings and unaffected control children.

Glutathione is a tripeptide of glutamate, glycine and cysteine and is the major intracellular redox (reduction/oxidation) buffer. The glutathione thiol/disulfide redox couple (GSH/GSSG) maintains the highly reduced intracellular microenvironment that is pivotal for effective antioxidant/detoxification capacity, redox-sensitive enzyme regulation, cell cycle progression (proliferation/differentiation), gene transcription of antioxidant response elements (ARE) and redox signaling (Biswas et al. 2006; Filomeni et al. 2002; Fratelli et al. 2005; Kwon et al. 2003; Pastore et al. 2003; Schafer and Buettner 2001). The GSH/GSSG redox status in plasma reflects intracellular hepatic metabolism where the vast majority of transsulfuration metabolism occurs. Cysteine is the rate-limiting amino acid for glutathione synthesis. The ratio of reduced cysteine (Cys) to its oxidized cystine disulfide form (CyS-S) represents the major extracellular redox couple that determines the redox status of specific cysteine residues in key regulatory cell surface proteins (Yan et al. 2009). The two major thiol/disulfide redox couples, GSH/GSSG and CyS/CyS-S, reversibly regulate the activation of key proteins by oxidative modification of sulfhydryl groups on exposed cysteine residues (Jones et al. 2004). Dynamic changes in status of glutathione and cysteine redox couples provide a "redox switch" that can regulate enzyme activation/inactivation, membrane signal transduction, cell adhesion and immune cell activation/proliferation (Biswas et al. 2006; Yan and Banerjee 2010). Oxidative stress/damage occurs when antioxidant defense mechanisms fail to counterbalance and control reactive oxygen species generated from endogenous oxidative metabolism or from pro-oxidant environmental exposures. Recent experimental studies and reviews support the hypothesis that chronic redox imbalance and resultant oxidative stress may be contributing factors to autism pathophysiology (Chauhan and Chauhan 2006; James et al. 2006; Kern and Jones 2006; Ming et al. 2005; Sogut et al. 2003; Vargas et al. 2005; Yao et al. 2006; Yorbik et al. 2002; Zoroglu et al. 2004). Based on the critical role of redox status in cell viability and function, we report for the first time the redox poise of both glutathione and cysteine redox couples as a reflection of systemic redox status in children with autism.

Because pathways of methionine transmethylation and transsulfuration are mutually interdependent, genetic or environmental perturbation of folate or methionine metabolism can indirectly impact cellular methylation capacity and glutathione synthesis. Conversely, genetic or environmentally-induced alterations in glutathione synthesis can alter flux through pathways of folate and methionine metabolism (Chan et al. 2008; Reed et al. 2008; Tchantchou et al. 2004; Vitvitsky et al. 2003). Viewed in the context of systems biology, these are core metabolic pathways that represent "hubs" for the regulation and modulation of cellular methylation, DNA synthesis and redox status in every mammalian cell. The biologic basis of autism is thought to involve gene-environment perturbations during critical developmental windows. It is therefore relevant to define the homeostasis of these polymorphic and environmentally sensitive pathways in children with autism.

In the present report, we build upon our previous findings in a new cohort of children participating in the autism *IMAGE* study (*I*ntegrated *M*etabolic *A*nd *G*enomic *E*ndeavor) that additionally provides a paired case–control comparison with unaffected siblings. Unaffected siblings represent an important new control group to determine whether the metabolic phenotype of the autistic children is specific for autism or whether it simply reflects shared genes and environmental exposures between siblings. In addition to replicating our previous report of systemic methylation and redox imbalance in plasma, we report for the first time genome-wide DNA hypomethylation (as percent 5-methylcytosine in DNA) and oxidative protein/DNA damage (as oxidized protein tyrosine derivatives and the oxidized DNA adducts) as evidence for a functional impact on epigenetic regulation and antioxidant/detoxification capacity in many children with autism.

Subjects and Methods

Participants

The autism IMAGE study (*I*ntegrated *M*etabolic *A*nd *G*enomic *E*ndeavor) is an on-going case–control study at Arkansas Children's Hospital Research Institute (ACHRI) that has recruited over 162 case and control families and is comprised of 68 case children, 40 unaffected siblings, and 54 age-matched unaffected control children. The autism case families were recruited locally after referral to the University of Arkansas for Medical Sciences (UAMS) Dennis Developmental Center and diagnosed by trained developmental pediatricians (ES, ML, JF, JB). Children 3–10 years of age with a diagnosis of Autistic Disorder as defined by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (*DSM-IV 299.0), the Autism Diagnostic Observation Schedule (ADOS), and/or the Childhood Autism Rating Scales (CARS > 30) were enrolled. The ADOS (Lord et al. 1989) and CARS

(Schopler et al. 1980) are diagnostic tools used to confirm the diagnosis and severity of autism. Children previously diagnosed with other conditions on the autism spectrum (pervasive developmental disorder-not-otherwise-specified (PDD-NOS), childhood disintegrative disorder or rare genetic diseases associated with symptoms of autism such as fragile X, Rett syndrome, or tuberous sclerosis were not included in the study. Children with chronic seizure disorders, recent infection, and high dose vitamin or mineral supplements above the RDA were excluded because these conditions are potential confounders effecting redox status. Unaffected siblings and unrelated neurotypical children ages 3–10 with no medical history of behavioral or neurologic abnormalities by parent report were the comparison groups. The unrelated neurotypical control children were was recruited by flyers placed in schools, clinics and at the University and received compensation for their participation. The mean ages of case children, unaffected siblings and unrelated control children was 5.8 ± 2.1, 5.6 ± 2.3, and 6.3 ± 2.1 years, respectively. The proportion of male children among case, sibling and control children was 85, 45, and 48%, respectively. The ethnic distribution among case families was 96% Caucasian, 2% Hispanic, and 2% Asian and among control families, the ethnic distribution was 90% Caucasian, 8% Hispanic and 2% Asian. Over-the-counter multivitamin supplement use at the time of blood draw was 35% among case children, 14% among siblings and 17% among the controls. The protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences and all parents signed informed consent.

Plasma Transmethylation and Transsulfuration Metabolites, 3-Nitrotyrosine and 3-Chlorotyrosine

Fasting blood samples were collected before 9:00 am into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 1,300×*g* for 10 min at 4°C. Aliquots of plasma were transferred into cryostat tubes and stored at −80°C until extraction and HPLC quantification. The methodological details for HPLC elution and electrochemical detection of these plasma metabolites were developed in our laboratory and have been described previously (Melnyk et al. 1999, 2000; Shigenaga et al. 1990). The storage interval at −80°C before extraction was consistently between 1 and 2 weeks after blood draw to minimize potential metabolite inter-conversion. Between-run variation was controlled by inclusion of internal standards with each run. Plasma total folate and vitamin B12 were measured using SimulTRAC-SNB Radioassay Kit for Vitamin B12/Folate from MP Biomedical, Inc. (Orangeburg, NY).

8-Oxo-Deoxyguanosine and Percent 5-Methylcystosine/Total Cytosine in DNA

DNA was extracted from whole blood using the Puregene DNA Purification kit (Qiagen, Valencia, CA). To ∼1 μg DNA, RNase A (Sigma, St. Louis, MO) was added to a final concentration of 0.02 mg/mL and incubated at 37°C for 15 min. The purified DNA was digested into component nucleotides using nuclease P_1, snake venom phosphodieasterase, and alkaline phosphatase as previously described in detail (Friso et al. 2002). DNA base separation and quantification of 5-methylcytosine and cytosine was performed with a Dionex HPLC–UV system coupled to an electrospray ionization (ESI) tandem mass spectrometer (Thermo-Finnigan LCQ) using a Phenomenex Gemini column (C18, 150 × 2.0 mm, 3 μm particle size) and expressed as percent 5-methylcytosine/total cytosine. The concentration of 8-oxo-deoxyguanosine in DNA was quantified with HPLC electrochemical detection (Helbock et al. 1998) and expressed as pmol/μg DNA.

Statistical Analyses

Metabolic data was compared using the Student's *t* test (S-Plus software, Seattle, WA) with significance set at 0.05. Given our a priori hypothesis based on previous results, correction for multiple comparisons was not implemented for case–control metabolite data. Case-sibling comparisons were done using the paired *t* test which tests the hypothesis that the mean difference between pairs is equal to zero. The paired *t* test is constructed by taking the mean difference of all observed pairs and dividing this by the standard error of all observed differences. The pairwise approach reduces variation and increases sensitivity because of the shared genes and environment between sibling pairs. Multiple regression analysis with S-Plus was used to evaluate relationships between metabolites.

Results

Transmethylation Metabolites, SAM/SAH Ratio and DNA Hypomethylation

In Table 1, the autism case children are compared to their unaffected paired sibling using the paired *t* test and cases and siblings are compared to unaffected control children using the standard Student's *t* test. Methionine is an essential amino acid that is transmethylated into homocysteine via SAM, the major cellular methyl donor. Both methionine and SAM were significantly decreased in children with autism compared to their paired unaffected siblings; however, there were no differences between siblings

Table 1 Plasma transmethylation metabolites and genome-wide DNA methylation (mean ± SD) of autistic case children, paired sibling and unaffected control children

Plasma metabolites	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
Methionine (μmol/L)	19.8 ± 2.6[a]	22.3 ± 4.3	<0.001	23.3 ± 3.9	ns
SAM (nmol/L)	61.6 ± 8.9[a]	70.7 ± 19.4	<0.006	71.0 ± 15.6	ns
SAH (nmol/L)	20.0 ± 4.6[a]	16.9 ± 3.9	<0.001	14.8 ± 4.1	0.05
Adenosine (μmol/L)	0.16 ± 0.07	0.11 ± 0.05	<0.001	0.14 ± 0.07	ns
Homocysteine (μmol/L)	4.86 ± 1.5	4.69 ± 1.0	ns	4.68 ± 1.0	ns
Folate (ng/ml)	19.9 ± 5.1	21.6 ± 4.1	ns	19.4 ± 4.2	ns
B12 (pg/ml)	872 ± 528	719 ± 288	ns	864 ± 552	ns
SAM/SAH	3.29 ± 1.1[a]	4.4 ± 1.7	<0.001	5.08 ± 1.8	ns
DNA methylation (%5mC)	3.03 ± 0.8[a]	3.9 ± 0.7	<0.001	4.13 ± 1.0	ns

* Case-sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

and unrelated age-matched controls. The methylation inhibitors, SAH and adenosine, were significantly increased in case children compared to their siblings. Sibling SAH levels were intermediate between case and control values and increased relative to controls. Plasma levels of homocysteine and the vitamin cofactors, folate and B12, were similar among all three groups. The SAM/SAH ratio, an indicator of methylation capacity, was decreased in children with autism compared to their siblings but not different between siblings and controls. Similarly, the percent 5-methylcytosine in DNA was decreased in case children but not in unaffected siblings and controls. Although Table 1 is limited to those case children with an unaffected sibling, the mean metabolite values of these 40 paired autism cases were not statistically different from the mean of the total IMAGE cohort of 68 case children. The children with autism were different from both siblings and unrelated controls for all metabolites except for homocysteine, folate and B12 which were not different between groups. There was no difference in the metabolite results between children who were taking over-the-counter vitamin supplements and those who did not.

Cysteine and Glutathione Metabolites and Redox Ratios

Cysteine, derived from methionine, is the rate limiting amino acid for glutathione synthesis. Total cysteine levels (protein-bound plus free) were lower in children with autism compared to their paired siblings ($p < 0.002$); however, siblings were not different from unrelated controls (Table 2).

Cystine (CyS-S), the oxidized disulfide form of cysteine, was significantly elevated in case children, but not their siblings who were also not different from controls. The free cysteine/cystine thiol redox couple (CyS/CyS-S) in plasma constitutes the major extracellular redox buffer. Shown for the first time, plasma free CyS/CyS-S redox ratio was significantly lower (more oxidized) in children with autism compared to their paired sibling whereas siblings were not

Table 2 Plasma redox metabolites (mean ± SD) of autistic case children, paired sibling and age-matched control children

Plasma metabolites	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
Total Cysteine (μmol/L)	189 ± 21[a]	203 ± 26	<0.002	212 ± 18	ns
Free Cysteine (μmol/L)	21.6 ± 6.45	22.5 ± 5.0	ns	23.6 ± 5.3	ns
Free Cystine (μmol/L)	34.1 ± 7.5a[a]	27.1 ± 8.7	<0.001	26.4 ± 5.7	ns
Free Cysteine/Cystine	0.68 ± 0.25[a]	0.89 ± 0.25	<0.001	0.93 ± 0.27	ns
GSH (μmol/L)	1.84 ± 0.40[a]	2.06 ± 0.41	<0.001	2.58 ± 0.79	<0.001
GSSG (μmol/L)	0.23 ± 0.10[a]	0.15 ± 0.08	<0.001	0.16 ± 0.07	ns
GSH/GSSG	9.45 ± 4.08[a]	17.4 ± 10.3	<0.001	18.3 ± 8.6	ns

* Case-sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

Table 3 Oxidized glutathione levels, protein oxidative damage and oxidative DNA damage (mean ± SD) of autistic case children, paired sibling and age-matched control children

Biomarkers of oxidative stress/damage	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
% Oxidized GSH (2GSSG/(GSH + 2GSSG))	22 ± 8.1[a]	12.7 ± 5.9	<0.001	11.4 ± 4.1	ns
3-Nitrotyrosine (nmol/L)	143 ± 74[a]	80 ± 43	<0.001	72 ± 27	ns
3-Chlorotyrosine (nmol/L)	51 ± 18[a]	34 ± 17	<0.001	26 ± 11	0.01
8-Oxo- deoxyguanosine (pmol/mg DNA)	95 ± 35[a]	65 ± 13	<0.001	63 ± 24	ns

* Case-Sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

different from unrelated controls. Plasma free glutathione levels were also decreased in case children compared to their paired siblings; however, mean sibling values were intermediate between cases and unaffected controls and significantly different than controls. Notably, both the major extracellular (CyS/CyS-S) and the major intracellular (GSH/GSSG) redox buffers were shifted to a more oxidized state in the children with autism compared to their siblings whereas the mean sibling values were not different from control values.

Percent Oxidized Glutathione and Protein/DNA Oxidative Damage

The percent oxidized glutathione, expressed as glutathione equivalents (2GSSG/(GSH + 2GSSG), is a comprehensive indicator of plasma and intracellular glutathione redox potential (Lenton et al. 1999). The case-sibling pair comparison was highly significantly different in contrast to the sibling-unrelated control comparison which was not different (Table 3).

Both 3-nitrotyrosine and 3-chlorotyrosine are stable oxidative post-translational modifications of protein tyrosine residues and both were significantly increased in plasma from children with autism compared to their paired siblings. In nuclear and mitochondrial DNA, 8-oxodG is a free radical-induced oxidative lesion that is widely used as a biomarker of oxidative damage. Table 3 shows the highly significant increase in leukocyte DNA 8-oxo-deoxyguanosine (8-oxo-dG) levels in children with autism compared to their siblings but no difference between unaffected siblings and unaffected control children.

Figures 2a and b are representative scatter plots that display individual data points of all 68 case and 54 unrelated control participants in the autism IMAGE study. There was a positive correlation between the two oxidized endpoints, nitrotyrosine and percent oxidized glutathione ($r = 0.50$), and a disparate distribution between cases and controls with control subjects clustered at the lower less oxidized end of the distribution (Fig. 2a). The negative correlation between glutathione concentration and oxidized nitrotyrosine levels ($r = -0.41$, Fig. 2b) is consistent with the protective antioxidant function of glutathione. Sibling values overlapped with the control values in both correlations (data not shown).

Discussion

An "endophenotype" can be a biochemical, neurologic, hormonal or immunologic biomarker that is influenced by both genes and environment and that is reproducibly associated with clinical symptoms of the disease (Gottesman and Gould 2003). As such, it can provide targeted clues to susceptibility alleles and targets of environmental vulnerability. Because an endophenotype is potentially modifiable, it could additionally provide insights into treatment strategies that can be followed longitudinally during intervention for clinical efficacy. The abnormal metabolic endophenotype we have observed in many autistic children may be due to subtle changes in gene products that regulate flux through folate and methionine dependent pathways or could reflect environmental exposures that perturb these pathways. Even small variations in gene expression and enzyme activity, if expressed chronically, can have a significant impact on downstream metabolic dynamics with functional consequences especially during pivotal developmental windows.

To determine whether the observed metabolic phenotype is specific to autism, metabolic profiles from proband-sibling pairs and unaffected control children were compared. Within our autism IMAGE cohort, the majority of metabolites in the methylation/redox pathways were significantly different between affected and unaffected siblings whereas unaffected siblings were not different from unrelated controls. These results suggest that these methylation/redox biomarkers are specific for autistic disorder and not present in unaffected siblings who share similar genes and environmental exposures. However, because the plasma samples were obtained after diagnosis, it is not

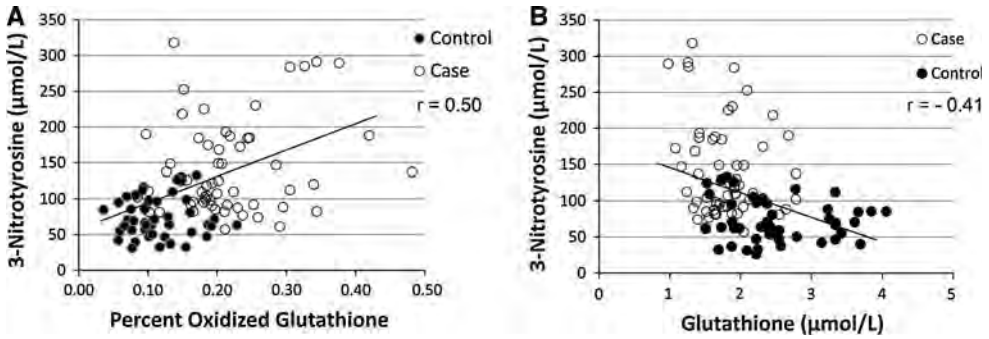

Fig. 2 **a** The positive correlation between the two oxidized endpoints, nitrotyrosine and percent oxidized glutathione ($r = 0.50$), and the disparate distribution between cases (*open circles*) and controls (*closed circles*) with control subjects clustered at the lower (less oxidized) end of the distribution. **b** The negative correlation between glutathione concentration and oxidized nitrotyrosine levels ($r = -0.41$) and is consistent with the protective antioxidant function of glutathione

possible to discern whether the observed deficits in methylation and redox capacity contribute to autism pathogenesis or are simply a reflection of on-going autism pathophysiology. Nonetheless, treatment to restore methylation and redox homeostasis has been shown to be efficacious in some children with autism and also in individuals with schizophrenia and bipolar disorder (Berk et al. 2008a, b; Dean et al. 2009; Dodd et al. 2008; Frankenburg 2007; James et al. 2009a; Strous et al. 2009). Although these studies suggest that targeted treatment can reverse the biochemical imbalance, further confirmation and randomized clinical trials are needed to determine whether restoration of metabolic balance can also improve medical and behavioral symptoms in children with autism.

The autism IMAGE study is our third independent case–control study that confirms findings of decreased plasma concentrations of methylation precursors and decreased SAM/SAH methylation potential in autistic children (James et al. 2006, 2009a). While reduced levels of metabolic precursors suggest reduced methylation capacity, here we report for the first time that genome-wide DNA methylation is also reduced in many autistic children. Although OTC vitamin use was higher in the autism group than in sibling and unaffected controls, the decrease in global DNA methylation observed was opposite of an expected effect. Further, statistical comparison of DNA methylation and plasma metabolites between children who were and were not taking OTC supplements revealed no significant differences in any group. Thus, we conclude that OTC vitamin use had no apparent effect on any of the endpoints measured. The presence of global DNA hypomethylation implies epigenetic dysregulation possibly due to reduced methyl donor (SAM) availability and/or increased product (SAH) inhibition of DNA and histone methyltransferases. A decrease in SAM/SAH methylation potential and DNA hypomethylation have been previously implicated in the etiology of cardiovascular disease, cancer, schizophrenia, autoimmunity and birth defects (Castro et al. 2003; Dunlevy et al. 2006; Li et al. 2010; Schmutte et al. 1996). Epigenetic modifications endow the genome with adaptive plasticity in gene expression in response to a changing environment and do so without altering DNA sequence. Primary epigenetic mechanisms include DNA cytosine methylation and histone methylation/acetylation that function to alter chromatin structure and transcription factor accessibility to initiate gene expression. Because DNA and histone methyltransferase reactions involve the transfer of a methyl group from SAM, epigenetics is integrally linked to transmethylation metabolism and methyl donor availability. While our observations of decreased methyl donor (SAM) and increased methyltransferase inhibitor (SAH) are consistent with epigenetic dysregulation, the functional significance of genome-wide DNA hypomethylation has yet to be defined.

Increasing evidence from both human and animal models suggest that aberrant DNA methylation during prenatal and early postnatal development might have a role in the etiology of autism (Allan et al. 2008; Jiang et al. 2004; Nagarajan et al. 2008; Samaco et al. 2005; Schanen 2006). DNA methylation is a mitotically heritable epigenetic mechanism that determines tissue-specific gene expression and silencing during pre-natal and post-natal neurodevelopment. Fetal DNA methylation patterns are established very early during embryogenesis and provide the basis for tissue-specific gene expression, allele-specific gene imprinting, X chromosome inactivation, and chromosome stability (Dean and Ferguson-Smith 2001; Reik 2007). Thus, aberrant epigenetic programming during critical periods of fetal development can result in aberrant timing of gene expression and cell differentiation that can heritably alter fetal phenotype (Zeisel 2009). In addition to mediating gene-environment interactions, epigenetic variation in gene expression might help to explain the broad

heterogeneity in symptom severity, onset and heritability within the autism spectrum. This provocative possibility requires further investigation.

Impaired methionine metabolism has been reproducibly associated with epigenetic dysregulation in several other neurobehavioral disorders (Costa et al. 2009; Graff and Mansuy 2009; Grayson et al. 2009; Krebs et al. 2009). Initial genome-wide epigenetic profiling in individuals with schizophrenia and bipolar disorder revealed DNA methylation changes that mapped to loci involving mitochondrial function, brain development as well as GABAergic and glutamatergic neurotransmission (Mill et al. 2008). Under pathologic progression, global DNA hypomethylation is often paradoxically associated with promoter-specific DNA hypermethylation and down-regulation of gene expression (Ehrlich 2002). Consistent with our observations of DNA hypomethylation in many children with autism, gene-specific alterations in DNA methylation of MeCP2 and oxytocin receptor genes have been recently reported in post-mortem autism brain (Gregory et al. 2009; Nagarajan et al. 2008). Together, these preliminary results support the possibility that epigenetic dysregulation contributes to aberrant gene expression in autism.

As indicated in Fig. 1, methionine transmethylation metabolism is integrally related to transsulfuration by providing metabolic precursors for cysteine and glutathione synthesis and in turn redox homeostasis. The autism IMAGE cohort replicates previous reports of a decrease in glutathione precursors and also a decrease GSH/GSSG redox status (James et al. 2006, 2009a). Plasma GSH/GSSG redox ratio is a reflection of intracellular redox homeostasis primarily due to the cellular export of GSSG to the plasma under conditions of intracellular oxidative stress (Eklow et al. 1981). Oxidized GSSG was significantly elevated among the autistic children compared to their siblings who were not different from unrelated controls. In addition, the IMAGE study provides new evidence for a decrease in extracellular free cysteine/cystine (CyS/CyS-S) redox poise and an increase in oxidative protein and DNA damage in many children with autism but not in their paired siblings. These findings are consistent with our hypothesis that transsulfuration metabolism in children with autism may not able to maintain redox homeostasis in the presence of chronic oxidative stress. Similar evidence of oxidative stress in blood, CSF and brain has been similarly reported in patients with schizophrenia and bipolar disorder (Andreazza et al. 2009; Dean et al. 2009). Total GSH concentration was decreased 27% in CSF and 52% in medial prefrontal cortex of schizophrenic patients (Do et al. 2000). Recently, a genetic and functional deficit in glutamylcysteine ligase (GCL), the rate limiting enzyme for glutathione synthesis, was associated with decreased GSH levels in fibroblasts derived from schizophrenic patients (Gysin et al. 2007). Although the age of onset is clearly different between schizophrenia and autism, the onset of both disorders coincides with critical time periods (infancy and adolescence) during which brain maturational processes and fine tuning of neuronal circuitry occurs (Giorgio et al. 2010; Guerri and Pascual 2010). Glutathione redox status is a pivotal determinant of oligodendrocyte and neuronal progenitor cell signaling in the branch point decision whether to differentiate, proliferate, or die (Noble et al. 2003). Thus, it is theoretically plausible that subtle redox imbalance during pivotal periods of brain cell maturation and development could negatively affect downstream brain cell trajectories, connectivity and synchronization in both schizophrenia and autism.

Plasma concentrations of oxidized protein tyrosine derivatives (3-nitrotyrosine and 3-chlorotyrosine) were increased in many of the autism IMAGE participants relative to paired siblings and control children. These post-translational modifications provide a stable biochemical footprint of oxidative stress and protein oxidative damage that can be followed longitudinally. Nitrotyrosine originates from protein tyrosines primarily via free radical attack by peroxynitrite, the highly reactive product of superoxide and nitric oxide. Chlorotyrosine is created by hypochlorous acid, a potent chlorinating oxidant derived from myeloperoxidase released by activated macrophages and neutrophils during an inflammatory response. As a marker of oxidative stress, elevated levels of nitrotyrosine have been found in alcoholics, smokers, diabetes, atherosclerosis, cystic fibrosis, and in pre-term infants (Mohiuddin et al. 2006). Within our autism cohort, nitrotyrosine was positively correlated with percent oxidized glutathione ($r = 0.50$; $p < 0.001$). Supporting our observations in plasma, a recent preliminary study found increased levels of nitrotyrosine in autism post-mortem brain (Sajdel-Sulkowska et al. 2009).

The 8-oxo-dG adduct in nuclear and mitochondrial DNA is a pre-mutagenic lesion that has been associated with oxidative DNA damage and the pathophysiology of aging, cancer and pro-oxidant environmental exposures (Pilger and Rudiger 2006). Once incorporated, 8-oxo-dG codes for error-prone DNA synthesis that promotes de novo mutations, repair-associated DNA strand breaks and genomic instability (Dahlmann et al. 2009). Elevated levels of 8-oxo-dG in DNA, plasma or urine is a commonly used biomarker for assessing oxidative DNA damage during inflammatory disease and pro-oxidant exposures. Our data indicating a significant increase in 8-oxo-dG concentration in peripheral lymphocyte DNA in children with autism is a new finding consistent with reduced glutathione-mediated antioxidant/detoxification capacity. The biological relevance of 8-oxo-dG is its established ability to induce G > T transversions which are common somatic mutations

under pro-oxidant conditions. While the functional relevance of this new finding is not clear, it provides substantial evidence for an oxidized nuclear microenvironment and DNA structural instability. In addition, it offers a possible mechanism for the multiple and variable de novo mutations that have been found to be present in children with autism but not their parents (Gauthier et al. 2009; Sebat et al. 2007; Smith et al. 2009).

Genomic instability and de novo mutations secondary to an oxidizing microenvironment can occur in parental germ cells, gestation and/or during early post-natal development in nuclear and/or mitochondrial DNA. Mitochondria are the primary source and target of reactive oxygen species and, lacking histones and DNA repair mechanisms, mitochondrial DNA is exceptionally vulnerable to oxidative damage. In an earlier study, we measured mitochondrial redox status in lymphoblastoid cells derived from autistic to control individuals and found that mitochondrial GSH/GSSG redox status was significantly more oxidized and accompanied by increased generation of reactive oxygen species (ROS) in autism compared to control cells (James et al. 2009b). These results together with the present data indicating oxidative DNA damage in primary leukocytes warrants further investigation into genomic instability, cellular and sub-cellular sequelae of oxidative stress in the autism brain.

It is generally agreed that the pathobiology of autism involves multiple and variable genetic and environmental factors that interact to increase risk of developing the disorder. Intense research effort has uncovered several potential targets of gene-environment interactions including impairments in mitochondrial function, synaptic connectivity, calcium channeling, excitation/inhibition and excessive glutamate among others. Attempts to coalesce these disparate clues into a unified conceptual framework or final common pathway have been the focus of several reviews and have yielded several plausible hypotheses that warrant further research investigation (Belmonte et al. 2004; Bourgeron 2009; Rubenstein 2010; Rubenstein and Merzenich 2003). Genetic and/or environmentally-induced alterations in folate-dependent one carbon metabolism (transmethylation and epigenetics) linked to methionine-dependent sulfur metabolism (transsulfuration and oxidative stress) offers another plausible framework that could connect some of these disparate functional correlates of autism. Epigenetic alterations and oxidative DNA damage observed in the present study are consistent with the gene-environment model proposed in the LEARn (Latent Early-life Associated Regulation) explanation for idiopathic neurobiologic diseases (Lahiri et al. 2009). The LEARn model proposes that genetic and environmental risk factors operate through alterations in DNA methylation and oxidative damage in susceptible genes resulting in altered gene expression. Unlike DNA sequence change, both redox and epigenetic alterations are dynamic adaptive responses to environmental stressors that are inherently reversible; thus, a deeper understanding of these alterations could not only provide new insights into the basic neurobiology of autism but could lead to novel targeted therapeutic strategies to treat and possibly prenatally prevent the development of autism.

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Research Article

Intracellular and Extracellular Redox Status and Free Radical Generation in Primary Immune Cells from Children with Autism

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The modulation of the redox microenvironment is an important regulator of immune cell activation and proliferation. To investigate immune cell redox status in autism we quantified the intracellular glutathione redox couple (GSH/GSSG) in resting peripheral blood mononuclear cells (PBMCs), activated monocytes and CD4 T cells and the extracellular cysteine/cystine redox couple in the plasma from 43 children with autism and 41 age-matched control children. Resting PBMCs and activated monocytes from children with autism exhibited significantly higher oxidized glutathione (GSSG) and percent oxidized glutathione equivalents and decreased glutathione redox status (GSH/GSSG). In activated CD4 T cells from children with autism, the percent oxidized glutathione equivalents were similarly increased, and GSH and GSH/GSSG were decreased. In the plasma, both glutathione and cysteine redox ratios were decreased in autistic compared to control children. Consistent with decreased intracellular and extracellular redox status, generation of free radicals was significantly elevated in lymphocytes from the autistic children. These data indicate primary immune cells from autistic children have a more oxidized intracellular and extracellular microenvironment and a deficit in glutathione-mediated redox/antioxidant capacity compared to control children. These results suggest that the loss of glutathione redox homeostasis and chronic oxidative stress may contribute to immune dysregulation in autism.

1. Introduction

Autism is a behaviorally defined neurodevelopmental disorder that usually presents in early childhood and is characterized by significant impairments in social interaction and communication and by abnormal repetitive hyper-focused behaviors. The prevalence of autism spectrum disorders has increased more than 10-fold in the last two decades, now affecting one in 110 US children, yet the etiology of these disorders remains elusive [1]. Glutathione depletion and oxidative stress have been implicated in the pathology of numerous neurobehavioral disorders including schizophrenia [2], bipolar disorder [3], and Alzheimer's disease [4]. Accumulating evidence suggests that redox imbalance and oxidative stress may also contribute to autism pathophysiology. Multiple biomarkers of oxidative stress have been identified in blood samples from children with autism [5–12]. Our group has reported a decrease in concentrations of glutathione (GSH) and several of its metabolic precursors, an increase in oxidized glutathione disulfide (GSSG), and a decrease in glutathione redox ratio (GSH/GSSG) in case-control evaluations of plasma and lymphoblastoid cell lines derived from children with autism [13–16]. Recently, several interactive polymorphisms in enzymes regulating glutathione synthesis were found to be more prevalent in children with autism suggesting that the glutathione deficit and predisposition to oxidative stress may be genetically based in some children [17].

Oxidative stress occurs when cellular antioxidant defense mechanisms fail to counterbalance endogenous ROS production and/or exogenous prooxidant environmental exposures. Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a tripeptide that functions as the major intracellular antioxidant and redox buffer against macromolecular oxidative damage. The glutathione thiol/disulfide redox couple (GSH/GSSG) is the predominant mechanism for maintaining the intracellular

microenvironment in a highly reduced state that is essential for antioxidant/detoxification capacity, redox enzyme regulation, cell cycle progression, and transcription of antioxidant response elements (ARE) [18–23]. Subtle variation in the relative concentrations of reduced and oxidized glutathione provides a dynamic redox signaling mechanism that regulates these vital cellular processes [24–27]. For example, in both CNS precursor cells and naïve immune cells, intracellular glutathione redox status is the primary determinant modulating the cellular decision to undergo cell cycle arrest, differentiation, or proliferation [27]. A reducing intracellular environment is required for proliferation, while a more oxidized microenvironment favors cell cycle arrest and differentiation. A chronic deficit in the GSH/GSSG redox ratio is considered to be a reliable indicator of oxidative stress and increased vulnerability to oxidative damage from prooxidant environmental exposures [28, 29].

In the extracellular plasma compartment, the cysteine/cystine (thiol/disulfide) redox couple independently provides the ambient redox environment for circulating immune cells. The ambient extracellular cysteine/cystine redox potential has been shown to be more oxidized than the intracellular GSH/GSSG redox potential and is independently regulated [30]. Dynamic shifts in the plasma cysteine/cystine redox potential alter the redox status of cysteine moieties in cell surface proteins to induce conformational changes in protein structure that can reversibly alter function [31, 32]. For example, under oxidizing extracellular conditions, redox-sensitive cysteine residues in the catalytic core of protein tyrosine phosphatases become oxidized and reversibly inactivate enzyme activity depending on the ambient cysteine/cystine redox potential [31, 33, 34]. Extracellular cysteine/cystine redox status is emerging as an important new signal transduction mechanism that can induce posttranslational alterations in downstream redox-sensitive proteins including a variety of enzymes, transcription factors, receptors, adhesion molecules, and membrane signaling proteins resulting in the dynamic modulation of their activity and function [32, 35, 36].

Recent studies have revealed numerous immunologic abnormalities among children with autism including alterations in immune cell proportions [37–40] and shifts in helper T-cell subpopulations after mitogenic stimulation [41, 42]. Peripheral blood mononuclear cells (PBMCs) from individuals with autism have been shown to produce higher levels of proinflammatory cytokines and abnormal levels of regulatory cytokines compared to control PBMCs at baseline and upon mitogenic stimulation [43–46]. Taken together, the immunological studies suggest a role for a dysregulated immune system in autism that potentially could be related to a deficit in glutathione-mediated antioxidant capacity and an oxidized microenvironment in immune cells. To investigate this possibility, we examined whether primary immune cells (PBMCs) from children with autism exhibit decreased intracellular glutathione redox capacity compared to PBMCs from age-matched control children and whether a more oxidized intracellular and extracellular microenvironment is associated with increased production of oxidizing intracellular free radicals. Because immune cells from children with autism have been shown to have abnormal responses to stimulation, we also elected to challenge the PBMCs with immune activators known to promote oxidative stress and measure the resulting intracellular glutathione redox status in activated isolated monocytes and T cells.

2. Subjects and Methods

2.1. Participants. This investigation was conducted on a subset of children from the autism IMAGE (Integrated Metabolic and Genomic Endeavor) study at Arkansas Children's Hospital Research Institute (ACHRI) that has recruited over 162 case and control families to date. The IMAGE cohort for this study consisted of 43 children diagnosed with autistic disorder and 41 unaffected control children (16 of which were unaffected siblings). The autism case families were recruited locally after referral to the University of Arkansas for Medical Sciences (UAMS), Dennis Developmental Center and diagnosed by trained developmental pediatricians. Children aged 3 to 10 with a diagnosis of autistic disorder as defined by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV 299.0), the Autism Diagnostic Observation Schedule (ADOS), and/or the Childhood Autism Rating Scales (CARS >30) were enrolled. Children diagnosed with other conditions on the autism spectrum or rare genetic diseases associated with symptoms of autism were excluded from the study. Children with chronic seizure disorders, recent infection, and high-dose vitamin or mineral supplements exceeding the RDA were also excluded because these conditions are potential confounders that could affect redox status. Unaffected siblings and unrelated, neurotypical children aged 3 to 10 with no medical history of behavioral or neurologic abnormalities by parent report made up the comparison group. The protocol was approved by the Institutional Review Board at UAMS, and all parents signed informed consent.

2.2. Materials. Culture flasks, plates, and pipettes were obtained from Corning Life Sciences (Lowell, Mass, USA). RPMI 1640, penicillin/streptomycin, Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), and glutamine were purchased from Life Technologies (Carlsbad, Calif, USA). Carboxy-H_2DCFDA (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl ester) was obtained from Molecular Probes (Carlsbad, Calif, USA). Human Monocyte Isolation Kit II and Human CD4 T cell Isolation Kit II were purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). Histopaque-1077 and all other chemicals were obtained from Sigma-Aldrich (St. Louis, Mo, USA).

2.3. Isolation of PBMCs and Stimulation of Monocytes and CD4 T Cells. Fasting blood samples ($\leq$20 mL) were collected before 9:00 AM into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 1300 $\times$g for 10 min at 4°C. Aliquots of plasma were stored at -80°C in cryostat tubes until extraction and HPLC quantification. PBMCs were isolated by centrifugation over Histopaque-1077. Red

blood cells were lysed using a brief (15 s) incubation with 1 mL ice-cold water. Approximately, 30×10^6 PBMCs were resuspended in RPMI 1640 medium (supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine) at a density of 10^6 cells/mL. Note that because we were unable to obtain 20 mL blood volume from every child, it was not possible to isolate and analyze monocytes and CD4 T cells for all participants. For monocyte stimulation, PBMCs were treated with 0.1 μg/mL lipopolysaccharide (LPS); for T-cell stimulation, PBMCs were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin. Cells were placed in a humidified 5% CO_2 incubator at 37°C for 4 hr. Stimulated monocytes and CD4 T cells were then isolated by negative selection using magnetic cell labeling as described by the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany). Using flow cytometry, we determined that ≥75% of isolated monocytes are positive for CD14 and that ≥87% of isolated CD4 T cells are positive for CD4. For HPLC quantification of GSH and GSSG, approximately 2×10^6 unstimulated (resting) PBMCs, stimulated monocytes, or stimulated CD4 T cells were pelleted, snap frozen on dry ice, and stored at −80°C.

2.4. Cell Extraction and HPLC Quantification of Intracellular Glutathione and Plasma Cysteine Redox Status. The storage interval at −80°C before extraction was consistently between 1-2 weeks after blood draw and cell isolation to minimize potential metabolite interconversion. The methodological details for intracellular and extracellular GSH extraction and HPLC elution and electrochemical detection have been described previously [15, 16], and metabolite detection does not require derivatization. Although most GSSG is present as a mixed disulfide with other thiols including cysteine, our measurements detect only the free GSSG in plasma. Glutathione and cysteine concentrations were calculated from peak areas of standard calibration curves using HPLC software. Intracellular results are expressed as nanomoles per milligram of protein using the BCA Protein Assay Kit (Pierce, Rockford, Ill, USA), and plasma results are expressed as micromoles per liter.

2.5. Measurement of Intracellular Free Radicals. Carboxy-H_2DCFDA (DCF) is a membrane-permeable ROS/RNS-sensitive probe that remains nonfluorescent until oxidized by intracellular free radicals. The intensity of DCF fluorescence is directly proportional to the level of free radical oxidation. Approximately, 10^6 PBMCs were resuspended in 1 mL RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine and stained in the dark for 20 min with 1 μM DCF at 37°C. Stained cells were washed and resuspended in PBS and analyzed immediately on a Partec CyFlow flow cytometer (Görlitz, Germany) using 488 nm excitation wavelength with 530/30 nm (FL1) emission filter. For each analysis, the fluorescence properties of 10000 cells were collected, and the data were analyzed using the FCS Express software (De Novo Software, Los Angeles, Calif, USA). Intracellular free radical levels are expressed as median fluorescence intensity (MFI) of subject sample DCF fluorescence normalized to DCF fluorescence of a standard PBMC preparation. As an internal control, the standard PBMC preparation was isolated from a 100 mL blood sample from an unaffected healthy adult volunteer, aliquoted and frozen at −180°C in 90% FBS/10% DMSO. An aliquot of the standard PBMC preparation was stained and analyzed with each subject sample. Evaluation of oxidizing free radical production was possible only in those case and unrelated control samples for which sufficient (~20 mL) blood volume was obtained.

Table 1: Demographics of study population.

	Case children $n = 43$	Control children $n = 41$
Age; mean (SD)	5.42 (1.98)	6.16 (2.29)
Male; *n* (%)	36 (84)	20 (49)
White; *n* (%)	38 (88.4)	31 (75.6)
Asian; *n* (%)	2 (4.65)	0 (0)
African American; *n* (%)	2 (4.65)	8 (19.5)
Hispanic; *n* (%)	1 (2.3)	2 (4.9)
OTC multivitamin use; *n* (%)	17 (39.5)	8 (19.5)

2.6. Statistical Analysis. Within the control group, 16 of the 41 unaffected control children were case siblings. There were 27 additional case children without a sibling and 25 additional unrelated control children comprising the total case-control cohort of 84 children. To down-weight the impact of outliers, three metabolites observations were curtailed at the extremes of the distributions for PBMC GSH, PBMC GSSG, and Monocytes GSH/GSSG (see footnote in Table 2). The sibling data are correlated resulting in a combined sample of correlated and uncorrelated data; thus, the assumption of all data being independent is not satisfied for the standard two-sample t-test. To make use of all data from dependent and independent observations, we used the corrected Z-test proposed by Looney and Jones [47]. This statistical approach provides adequate control of Type 1 errors and has more power than a standard Student's t-test. Because the DCF data compared cases and unrelated controls (without siblings) the standard Student's t-test was used with significance set at 0.05. Nonparametric intercorrelations (Spearman correlation coefficients) between age and gender and the 7 outcome variables, GSH, GSSG, GSH/GSSG, % oxidized glutathione, cysteine, cystine, and cysteine/cystine were determined with the significance level set at 0.05. Data was analyzed using SAS 9.2 software (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Demographics of Study Population. Table 1 lists the demographics of the study population. The only major differences between cases and controls are that the control group was composed of a greater proportion of females and African Americans, whereas the case group had a greater proportion of Asian subjects. Over-the-counter multivitamin supplement use was higher among cases (39.5%) compared

Table 2: Intracellular glutathione redox status in resting PBMCs and activated monocytes and CD4 T cells.

Metabolite	Case children		Control children		Corrected Z-test	
	n	Mean ± SD	n	Mean ± SD	Difference (95% CI)	P value
Resting PBMCs						
GSH (nmol/mg protein)	43	25.45 ± 8.16	41	23.35 ± 6.38	2.09 (−1.09, 5.29)	0.19
GSSG (nmol/mg protein)	43	0.90 ± 0.3	41	0.66 ± 0.23	0.24 (0.13, 0.35)	<0.001
GSH/GSSG	43	29.58 ± 9.04	41	37.58 ± 10.89	−7.99 (−12.51, −3.48)	<0.001
Oxidized GSH (%)	43	0.07 ± 0.02	41	0.05 ± 0.01	0.02 (0.0075, 0.024)	<0.001
Activated monocytes						
GSH (nmol/mg protein)	18	7.73 ± 3.16	20	8.55 ± 2.5	−0.82 (−2.02, 0.38)	0.18
GSSG (nmol/mg protein)	18	0.62 ± 0.24	20	0.47 ± 0.17	0.14 (0.03, 0.25)	0.01
GSH/GSSG	18	13.31 ± 7.26	20	19.30 ± 6.35	−5.98 (−9.99, −1.97)	0.003
Oxidized GSH (%)	18	0.14 ± 0.05	20	0.10 ± 0.03	0.04 (0.02, 0.07)	<0.001
Activated CD4 T cells						
GSH (nmol/mg protein)	18	6.82 ± 3.0	19	10.16 ± 3.74	−3.33 (−5.24, −1.42)	<0.001
GSSG (nmol/mg protein)	18	0.68 ± 0.29	19	0.63 ± 0.24	0.05 (−0.11, 0.22)	0.51
GSH/GSSG	18	10.47 ± 4.19	19	17.49 ± 6.95	−7.02 (−10.17, −3.87)	<0.001
Oxidized GSH (%)	18	0.17 ± 0.05	19	0.11 ± 0.05	0.05 (0.03, 0.08)	<0.001

GSH: glutathione; GSSG: oxidized glutathione disulfide; oxidized GSH: (%)2GSSG/(GSH+2GSSG); curtailment: PBMC GSH >45 set = 45 ($n = 1$); PBMC GSSG >1.75 set = 1.75 ($n = 1$); Monocytes GSH/GSSG >35 set = 35 ($n = 1$).

to controls (19.5%); however, the glutathione redox status was statistically unaffected by vitamin use (data not shown).

3.2. Decreased Intracellular Glutathione Redox Status in Autism. Table 2 presents the relative intracellular concentrations of GSH, GSSG, the glutathione redox ratio, and the percentage of oxidized glutathione equivalents in resting (unstim-ulated) PBMCs and in isolated stimulated monocytes and CD4 T cells from children with autism and age-matched control children. The percent oxidized glutathione is expressed in absolute glutathione equivalents as 2GSSG/(GSH+2GSSG). Relative to controls, the intracellular concentration of GSSG and the percent oxidized glutathione were significantly increased (∼40%), and the GSH/GSSG ratio decreased (∼21%) in PBMCs from children with autism ($P < 0.001$). After stimulation with LPS, monocytes from children with autism also exhibited significantly decreased GSH/GSSG (∼31%, $P = 0.003$), increased GSSG concentration (∼32%, $P = 0.01$), and 40% higher percent oxidized glutathione ($P < 0.001$). In mitogen-stimulated CD4 T cells from children with autism, the intracellular GSH concentration was ∼33% lower, the GSH/GSSG was ∼40% lower ($P < 0.001$), and the percent oxidized glutathione was ∼55% higher than in stimulated CD4 T cells from control children (<0.001). As expected, activation with LPS and PMA both resulted in decreased intracellular GSH levels and GSH/GSSG in isolated monocytes and CD4 T cells compared to resting (unstimulated) PBMCs. Upon stimulation, there was a greater decrease in intracellular GSH and GSH/GSSG in both CD4 T cells and monocytes from children with autism compared to control children. Neither age nor gender was significantly correlated with any of the outcome measures. The protein content per 10^6 cells did not differ between case and control children (data not shown).

3.3. Decreased Extracellular Glutathione and Cysteine Redox Status in Autism. Table 3 presents the relative concentrations of GSH, GSSG, GSH/GSSG, % oxidized GSH, cysteine, cystine, and the cysteine/cystine redox ratio in the extracellular plasma compartment. Children with autism exhibited a significantly decreased extracellular concentration of GSH (∼21%) and GSH/GSSG (∼54%) and increased concentration of GSSG and the percent oxidized glutathione (52% and 82%, resp., $P < 0.001$). Figures 1(a) and 1(b) compare GSH/GSSG and % oxidized glutathione equivalents, respectively, in plasma, T cells, and monocytes from case and control children and graphically demonstrates the consistent decrease in both extracellular and intracellular glutathione redox status among the case children.

The dynamic balance between the reduced and oxidized forms of glutathione can also be expressed as the redox potential or reducing power of the GSH/GSSG redox couple (E_h) and can be calculated from the Nernst equation, $E_h = E_0 + RT/nF \ln[\text{disulfide}]/([\text{thiol}\,1] * [\text{thiol}\,2])$, where E_0 is the standard potential for the glutathione redox couple (−264 mV), R is the gas constant (8.314 J/°Kmol), T is the absolute temperature of analytical measurement (25°C = 298°K), n is 2 for the number of electrons transferred, and F is Faraday's constant (96,485 coulomb/mol) [48]. The calculated E_h value for the GSH pool in the children with autism is −116 mV, which is 12 mV more oxidized than in the control children, with an E_h value of −128 mV (Table 3).

Table 3: Extracellular (plasma) glutathione and cysteine redox status.

Metabolite	Case children		Control children		Corrected Z-test	
	n	Mean ± SD	n	Mean ± SD	Difference (95% CI)	P value
Plasma						
GSH (μM)	38	1.58 ± 0.23	41	1.99 ± 0.22	−0.41 (−0.50, −0.31)	<0.001
GSSG (μM)	38	0.20 ± 0.06	41	0.13 ± 0.04	0.07 (0.05, 0.09)	<0.001
GSH/GSSG	38	8.24 ± 2.20	41	17.14 ± 5.54	−8.73 (−10.52, −6.94)	<0.001
Oxidized GSH (%)	38	0.20 ± 0.05	41	0.11 ± 0.03	0.09 (0.07, 0.10)	<0.001
E_h for GSH		−116 mV		−128 mV		
Cysteine (μM)	41	21.7 ± 4.88	41	21.43 ± 4.08	0.13 (−1.88, 2.14)	0.90
Cystine (μM)	41	29.2 ± 10.6	41	19.26 ± 4.8	9.73 (6.25, 13.2)	<0.001
Cysteine/Cystine	41	0.79 ± 0.18	41	1.14 ± 0.18	−0.33 (−0.41, −0.26)	<0.001
E_h for Cysteine		−106 mV		−111 mV		

GSH: glutathione; GSSG: oxidized glutathione disulfide; E_h: steady-state redox potential; E_h for GSH: $-264\,\text{mV} + (30\,\text{mV}) * \log([\text{GSSG}]/[\text{GSH}]^2)$; E_h for cysteine: $-250\,\text{mV} + (30\,\text{mV}) * \log([\text{CySSCy}]/[\text{Cys}]^2)$.

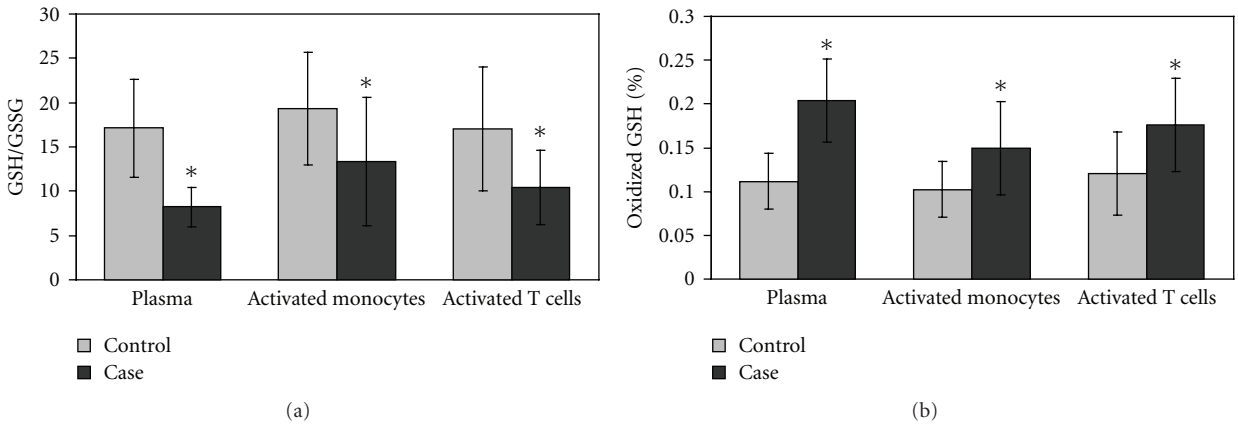

Figure 1: Intracellular and extracellular glutathione redox imbalance in autism. (a) presents the GSH/GSSG in plasma, isolated activated monocytes, and CD4 T cells from case and control children; (b) presents the % oxidized glutathione equivalents. Both extracellular and intracellular glutathione redox status are consistently significantly decreased among the case children ($^*P < 0.01$).

The concentration of cystine, the oxidized form of cysteine, was significantly elevated (~52%), while the cysteine/cystine redox ratio was significantly decreased (~31%) in plasma from children with autism ($P < 0.001$). The E_h value for the cysteine pool can also be calculated from the Nernst equation (see above) where the E_0 for cysteine is equal to −250 mV [30]. The calculated E_h value for the cysteine pool in children with autism is −106 mV, or 5 mV more oxidized than the control E_h value of −111 mV (Table 3).

3.4. Elevated Free Radical Production in Autism. The level of intracellular free radicals was measured in available resting PBMCs from children with autism ($n = 15$) and unaffected control children ($n = 16$) using DCF, an ROS/RNS-sensitive fluorescent probe. Monocytes and lymphocytes were gated based on light scatter properties (size and density) and analyzed separately. Figure 2 presents the median fluorescence intensity (MFI) of lymphocytes from children with autism and unaffected control children (normalized to MFI of the standard PBMC preparation). Gated lymphocytes from children with autism exhibited a significantly higher mean level of intracellular free radicals compared to lymphocytes from control children ($P < 0.05$). No differences in free radical production were observed in gated monocytes from case and control children. Intracellular free radical production was not correlated with age or gender in this cohort.

4. Discussion

Oxidative stress is generally defined as an imbalance between oxidant production and endogenous antioxidant defense mechanisms and can be clinically defined in humans by a decrease in the redox status of GSH/GSSG and cysteine/cystine thiol/disulfide redox couples [49]. The relative equilibrium between reduced and oxidized sulfhydryl groups defines the ambient redox state. Low glutathione redox status

has been associated with the pathophysiology of several neurobehavioral disorders including schizophrenia [2, 50], bipolar disorder [3], alcoholism [51], HIV [52], and Alzheimer's disease [53]. This is the first study to evaluate intracellular glutathione-mediated antioxidant/redox capacity in primary cells from children with autism as well as the extracellular plasma cysteine/cystine redox status. Because these two redox systems are compartmentalized and independently regulated, evaluation of both redox couples provides a complete picture of the primary immune cell microenvironment in children with autism. Supporting and extending our previous findings of decreased plasma and lymphoblastoid cell GSH/GSSG, we now report that both primary immune cell GSH/GSSG and plasma cysteine/cystine redox couples are similarly compromised resulting in a more oxidized immune cell microenvironment in children with autism compared to control children.

Recent evidence supports the notion that subtle fluctuations in ambient redox status may provide an important regulatory mechanism that can dynamically modulate immune cell function. Activation and proliferation of T cells require a reducing intracellular microenvironment, whereas a more oxidized environment promotes cell cycle arrest and blunted responsiveness to immune stimulation [54–57]. For example, a mechanism involving extracellular redox modulation by regulatory T cells (Tregs) was recently elucidated by Yan et al. [35]. Tregs were shown to inhibit the release of cysteine into the immune synapse between dendritic cells and naïve T cells, which effectively reduces GSH levels in T cells by eliminating the rate-limiting amino acid for GSH synthesis. A high ratio of reduced to oxidized glutathione is required for cell cycle progression from G1 to S phase and induction of the T-cell proliferative response [55]. Thus, the more oxidized GSH/GSSG redox state of the intracellular glutathione pool in PBMCs and in activated CD4 T cells observed in children with autism (Table 2) would suggest a hyporesponsive phenotype that is less conducive to T-cell activation and proliferation. Consistent with this hypothesis, several recent studies have documented abnormalities in the adaptive immune response in children with autism [44, 58].

A glutathione deficit in T cells has been shown to negatively affect the adaptive immune response and T-cell proliferation by reducing IL-2 receptor turnover and IL-2-dependent DNA synthesis [59, 60]. In monocytes, an oxidized intracellular environment has been shown to alter the cytokine profile and skew the Th1 and Th2 balance [61, 62]. Studies in mice have demonstrated that the intracellular GSH content of antigen presenting cells (APCs) reversibly alters the Th1 and Th2 cytokine response pattern [61]. Specifically, a GSH deficit reduced Th1-associated IFN-γ production and exaggerated Th2-associated IL-4 production. Restoration of GSH restored the Th1 cytokine response and normalized the Th2 response. Consistent with these observations, two independent studies have reported that helper T-cell subpopulations in PBMCs from children with autism are shifted towards T helper 2 (Th2) dominance [41, 42]. Further, a decrease in T-cell IL-2 receptor expression has been reported

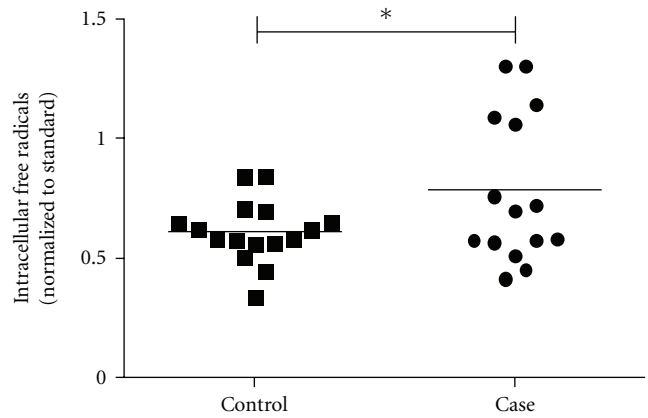

Figure 2: Intracellular Free Radicals are Elevated in Lymphocytes from Children with Autism. Intracellular free radicals were measured in freshly isolated PBMC from children with autism and unaffected control children using 1 uM DCF. Presented is median fluorescent intensity (MFI) of the gated lymphocyte population from subject samples normalized to MFI of a standard PBMC preparation also treated with 1 uM DCF and analyzed with each subject sample. Lymphocytes from children with autism exhibited a significantly higher mean level of intracellular free radicals than controls ($P = 0.04$). Control median (95% CI) = 0.576 (0.551–0.640); case median (95% CI) = 0.689 (0.561–1.086).

to be associated with decreased proliferative response after mitogen stimulation in children with autism [58].

The more oxidized GSH/GSSG redox status in plasma and primary immune cells in children with autism (Figure 1) may offer a mechanistic explanation for the abnormal adaptive immune response previously reported in these children. When intracellular oxidative stress exceeds glutathione redox capacity, cells export GSSG into the plasma as a mechanism to restore internal redox homeostasis [49, 63]. The elevated GSSG concentrations in PBMCs (Table 2) suggest that the GSSG export mechanism and intracellular antioxidant capacity were not sufficient to maintain intracellular redox homeostasis and that redox imbalance was chronic in these children. The association between a more oxidized immune cell microenvironment and an abnormal adaptive immune response warrants continued investigation especially in light of the potential reversibility of immune dysfunction with targeted treatment to restore redox homeostasis [15].

The calculated E_h values for the extracellular GSH and cysteine pools (Table 3) in our control population differ somewhat from previously published values. In adults, the plasma glutathione E_h is more reduced at around -137 mV, and the plasma cysteine redox couple is more oxidized at -80 mV [30, 48]. These discrepancies may reflect methodological differences in sample preparation in that our electrochemical detection does not require derivatization for detection. It is also possible that children (age 3–10 years) may have less reducing capacity than previously reported in adults (age 25–35 years) [48]. Nonetheless, our calculated E_h values are consistent with previous reports that plasma cysteine E_h (-111 mV) is more oxidized than that of GSH (-128 mv).

Mean intracellular free radical production was higher in primary lymphocytes from children with autism relative to lymphocytes from age-matched control children (Figure 2) and was driven by a subset of 5 (33.3%) children whose lymphocytes exhibited especially high levels of free radicals. Mitochondria are the primary producers and targets of intracellular free radicals, and mitochondrial dysfunction has been postulated to be a contributing factor in the pathogenesis of autism and numerous other neurological disorders [64–67]. In a lymphoblastoid cell model, we previously demonstrated that the GSH/GSSG redox ratio in mitochondria was significantly lower in autism compared to control cells and was associated with a significantly lower mitochondrial membrane potential after nitrosative stress [16]. It is well established that mitochondria are highly concentrated in presynaptic terminals and that loss of redox control can negatively affect the efficiency of neurotransmission and synaptic plasticity [68, 69]. Similarly, mitochondrial localization and redox signaling at the immunological synapse between lymphocytes and antigen presenting cells are required for immune activation, and excessive ROS can interrupt these signaling pathways [70–72]. A recent study of mitochondrial defects in lymphocytes from children with autism found decreased complex I activity and overreplication of and deletions in mitochondrial DNA compared to control lymphocytes [73]. Based on this evidence, it is plausible to hypothesize that mitochondria may be the source of the increased levels of lymphocyte free radicals observed in the subset of autistic children presented in Figure 2. Consistent with this hypothesis, a recent meta-analysis estimated that mitochondria dysfunction may affect up to 30% of children with autism [64]. Based on this evidence, further study of mitochondrial function and redox status in lymphocytes from children with autism is warranted.

Relevant to our observations, two recent papers have revealed that an oxidized extracellular cysteine/cystine redox status can initiate a redox signaling cascade that stimulates intracellular mitochondrial ROS production as a mechanism to initiate an inflammatory immune response [74, 75]. The signal transduction from the extracellular to intracellular compartments occurs through oxidative modification of redox-reactive cysteines on cell surface proteins. Exposed cysteine sulfhydryl groups on proteins can be reversibly oxidized to sulfenic acid or disulfide bonds resulting in altered protein structure and function that initiate downstream redox signaling cascades [33, 76, 77]. In an elegant series of experiments, Imhoff and Hansen demonstrated that mitochondrial ROS production was significantly increased in cells incubated under extracellular oxidized cysteine/cystine redox conditions [74]. The stimulated intracellular ROS production resulted in the expression of Nrf-2, the transcription factor responsible for initiation of the inflammatory response. Treatment to block the availability of cell surface cysteine thiol groups abrogated mitochondrial ROS production and Nrf-2 expression. Go et al. confirmed and extended these observations by demonstrating that treatment to maintain mitochondrial redox status abrogated ROS production in the presence of oxidized extracellular cysteine/cystine [75]. Although the precise mechanism for the oxidative cysteine/cystine-dependent signaling for mitochondrial ROS production is not yet clear; the authors provide evidence of a possible link to changes in the redox state of cytoskeletal proteins that could be functionally linked to the mitochondrial membrane. Other studies have demonstrated that an oxidized plasma cysteine/cystine redox potential is associated with proinflammatory conditions [78, 79] and can be modulated by diet [80, 81]. These observations support the possibility that the oxidized plasma cysteine/cystine in children with autism may be functionally related to the increase in lymphocyte free radical production observed and contribute to immune cell abnormalities in these children.

In summary, we show for the first time that both the extracellular and intracellular immune cell compartments are more oxidized in children with autism compared to age-matched unaffected control children. Randomized clinical trials will be needed to determine whether treatment to normalize plasma and intracellular redox status will improve immune cell function and possibly the health and behavior in children with autism.

Conflict of Interests

The authors declare no conflict of interests.

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